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# What are the scientific challenges in moving from targeted to non-targeted methods for food fraud testing and how can they be addressed? – Spectroscopy case study

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ABSTRACT

*Background:* The authenticity of foodstuffs and associated fraud has become an important area. It is estimated that global food fraud costs approximately $US49b annually. In relation to testing for this malpractice, analytical technologies exist to detect fraud but are usually expensive and lab based. However, recently there has been a move towards non-targeted methods as means for detecting food fraud but the question arises if these techniques will ever be accepted as routine.

*Scope and approach:* In this opinion paper, many aspects relating to the role of non-targeted spectroscopy based methods for food fraud detection are considered: (i) a review of the current non-targeted spectroscopic methods to include the general differences with targeted techniques; (ii) overview of in-house validation procedures including samples, data processing and chemometric techniques with a view to recommending a harmonized procedure; (iii) quality assessments including QC samples, ring trials and reference materials; (iv) use of "big data" including recording, validation, sharing and joint usage of databases.

*Key findings and conclusions:* In order to keep pace with those who perpetrate food fraud there is clearly a need for robust and reliable non-targeted methods that are available to many stakeholders. Key challenges faced by the research and routine testing communities include: a lack of guidelines and legislation governing both the development and validation of non-targeted methodologies, no common definition of terms, difficulty in obtaining authentic samples with full traceability for model building; the lack of a single chemometric modelling software that offers all the algorithms required by developers.

## 1. Introduction

Food fraud, in one guise or another, has been documented in literature since the times of the ancient Greeks. However, in more recent times, food fraud has garnered much greater notoriety due to a variety of factors: the growing complexity of food supply chains and the substantially greater opportunities to conduct fraud across them; and the advances made in this information rich age where news stories are instantly accessible and shared around the world. Globalisation of the food supply chain has far reaching impacts when adulteration occurs as witnessed with the Chinese milk Scandal in 2008 (Gossner et al., 2009) and the 2013 horsemeat scandal (Barnett et al., 2016). These scandals have helped refocus attention on developing measures to ensure the integrity of the food supply chain, with an increase in demand for food fraud detection to be proactive, rapid and reliable to maintain the security of the food chain as well as acting as a deterrent (Ellis et al., 2012). Food fraud often involves economically motivated adulteration, with unscrupulous producers aiming to increase profit margin by any means necessary with little regard for consumer safety, with the subsequent use of unconventional and in some cases non-food adulterants which are unlikely to be detected using the conventional targeted analysis (Moore, Spink, & Lipp, 2012). This has brought about the necessity to develop non-targeted testing systems which encapsulates the chemical analysis of the whole food matrix leading to the development of a food fingerprint as opposed to targeted analysis where a known analyte is specifically screened for (Riedl, Esslinger, & Fauhl-Hassek, 2015).

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### 1.1. Targeted and non-targeted analysis

Testing for food adulteration has become increasingly difficult due to the multitude of possible substances which can, and have been, used for adulterating foodstuffs. Targeted food analysis involves identifying specific marker compounds which are indicative of a particular property, with results compared to established limits to determine if the compound tested is in excess (Esslinger, Riedl, & Fauhl-Hassek, 2014). This poses a serious weakness for targeted analysis since the modus operandi of food fraudsters is often based on avoiding food testing and in many cases are sophisticated and have the knowledge of such testing programmes and how to avoid detection.

Non-targeted analysis, for food fraud, is comprised of using an analytical technique that affords a detailed profile of the representative authentic sample. While the profile may vary depending on the technique, it will be possible to compare the profile of the sample in question to a library of profiles gathered previously that represent historic material that has been shown to be fit for purpose. Appropriate statistics can then be used to discern if there is a difference between the new and historic samples (Ellis et al., 2012). The question that is posed during non-targeted analysis for food authenticity is; is this product authentic? i.e. fits within the population of authentic samples. In some cases this is all the data that will be obtained while in others information of which adulterant is present might be evident. Fig. 1 provides an overview of the stages involved in non-targeted analysis.

There are numerous non-targeted techniques currently available including vibrational spectroscopic methods (such as Fourier transform infrared (FT-IR), near infrared (NIR), hyperspectral imaging (HSI) and Raman) and nuclear magnetic resonance (NMR), spectrometry and chromatography based technologies. Vibrational spectroscopic techniques offer a rapid, high throughput and non-destructive method of analysis which is necessary for effective management of a fast-paced global food network. Furthermore, these technologies require only a limited amount of training for processing, making them user friendly and accessible for use in the field or on the production line (Ellis et al., 2012). In spectroscopy, following the collection of scans or “fingerprints”, results are evaluated using chemometric models as the raw spectra are too complicated to process visually. Chemometric models extract the important information that distinguishes different clusters therefore ignores redundant data and simplifies this process. Chemometrics utilises mathematical and statistical modelling to recognise patterns and relationships within highly complex data and translate them into useable analytical parameters. Patterns are identified within the results and then classified based on the relationship between the data. These can be used to identify food samples based on geographical origin, species variety as well as highlighting the contamination and adulteration of a sample. Much research has been undertaken regarding food fingerprinting methods with results demonstrating the feasibility of non-targeted testing approaches for food fraud screening. However, uptake in terms of routine surveillance has been limited to date (Riedl et al., 2015). This can be in large parts attributed to a lack of standardisation and validation of such methodologies, which is necessary to guarantee reliable and reproducible results, or current legislation that requires a targeted approach that qualifies and quantifies an adulterant in order for it to be legally incontestable. The performance of targeted analytical methodologies detecting certain substances and residues in live animals and animal products are covered by EU legislation (Commission Decision 2002/657/EC) of which there is no equivalent legislation as yet for non-targeted testing. To become truly standardised the issue of acceptance of this kind of testing in legislation must be addressed before these methods can be widely applied in practice. Cost has often been quoted as another barrier to implementation. However there is a broad range in instrument prices, ranging from a few hundred pounds up and into the hundreds of thousands of pounds, and researchers are actively working on non-targeted methodologies covering all these price points. Beyond the cost of the instrument, much of the expense is at the research and development stage where operators skilled in experimental design, sample collection and preparation, spectroscopic techniques and chemometric methods are required to develop and maintain the methods. Those developing and those using the methods are not necessarily the same people. The cost of the research can be recouped through commercialisation of techniques that are fully developed and validated whereby end users are simply paying for access to the sample protocols and model interface.

Examples of commercial applications that have been described as non-targeted methodologies can be found in the dairy industry, namely the DairyGuard by Perkin Elmer (Perkin Elmer, 2017) which works by comparing unknown samples against a library containing spectra of unadulterated and known adulterant materials; and the Abnormal spectrum screening module (ASM) from Foss (Foss, 2014) which is based on principal component analysis to identify spectra that are abnormal therefore should undergo further investigation.

The aim of this article is to review the non-targeted food fraud approaches, using spectroscopic techniques, currently reported in the literature with emphasis on method development, detection capabilities and validation robustness. Furthermore, the sample preparation, statistical modelling, sensitivities claimed will be assessed for common approaches. Finally, the challenges and recommendations for moving from targeted to non-targeted testing for food fraud detection will be identified. For the purpose of this scientific opinion, traditional

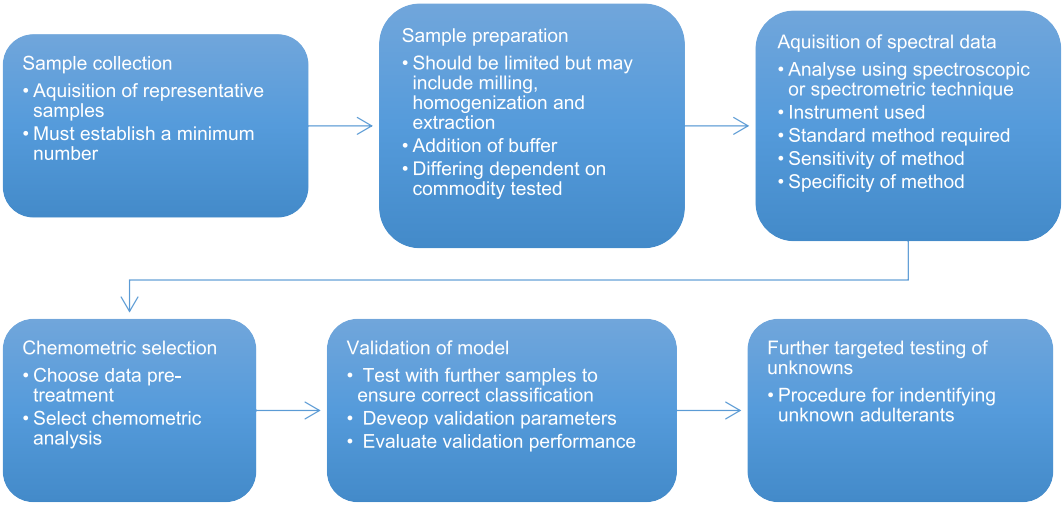


**Fig. 1.** Schematic overview of the stages involved in non-targeted analysis.

vibrational spectroscopy (NIR, FT-IR and Raman), hyperspectral imaging and NMR spectroscopy have been separated due to the differences in physical characteristics, sample preparation and commodities investigated.

## 2. Traditional vibrational spectroscopies

For technical details on FT-IR, NIR and Raman, the reader is directed towards the numerous papers cited in this opinion.

### 2.1. Literature search and eligibility criteria

The reader is directed to the supplementary information for details concerning the literature search and eligibility criteria.

### 2.2. Review of method development and validation for beef, milk and olive oil

In 2017, the United States Pharmacopeial Convention published guidelines on developing and validating non-targeted methods for food adulteration detection (U.S. Pharmacopeial Convention, 2017). These guidelines were not intended to cover the use of multi-class classification methods however many of the suggestions remain viable to the assessment of such methods performance. Likewise, they state that methods that return a concentration of a known adulterant should be considered a targeted method. Furthermore a scientific concept for validation of two class models was published (Alewijn, van der Voet, & van Ruth, 2016). For the purpose of clarity within this scientific opinion we have adopted the USP nomenclature for terms and provide clarification with a definition, where appropriate. We have taken the appropriate guidelines and expanded to some degree and added additional criteria we thought to be relevant.

This Scientific Opinion identifies and assesses the following criteria for non-targeted food fraud detection in beef, milk and olive oil, which are the commodities with the most publications meeting the eligibility criteria, with the belief that these principles can be applied to other food commodities:

- Objective of methodology
- Adulterant investigated
- Sample related criteria
- Instrumentation methodology
- Chemometric methodology
- Model performance criteria

Based on the evaluation of these criteria, a list of challenges and recommendations have been developed.

### 2.3. Results and discussion

#### 2.3.1. A review of non-targeted methods (NIR, FT-IT & Raman in conjunction with multivariate chemometric techniques) used in food authenticity

*2.3.1.1. Commodities covered and number of publications.* The literature searches undertaken identified 16 general commodity groupings that could be further divided into a total of 40 sub-commodities, Fig. 2 and Table 1. It is evident that NIR, FT-IR and RAMAN spectroscopies have been used to detect food fraud issues for a wide variety of commodities, especially over the last 6 years. In total 112 papers were found to fit the eligibility criteria, Table 1 (the list of references are available in Supplementary Table 1).

Fig. 2 and Table 1, show that oil has the most diverse coverage in terms of sub-commodities (11) investigated for fraud related activities, with most articles focusing on olive oil (8 out of 22 articles). This is not unexpected as extra virgin olive oil is a well-known and often publicised target for fraudsters. Although there are fewer dairy products covered (butter, cheese, milk & milk powder and cream) than oil, it is the commodity grouping with the most research articles (32 articles) covering the detection of fraud related questions using NIR, FT-IR or Raman spectroscopies. The majority of these articles (20 articles) are concerned with milk or powdered milk, Table 1. Based on the criteria for the literature research, developing methods, using spectroscopy and chemometric methodology, to detect fraud activities in the meat commodity grouping is the third largest research area in terms of the number of published papers (16 articles). The majority focused on beef authenticity/adulteration (12 articles) as expected with the time frame of the literature search including the 2013 European horsemeat scandal.

*2.3.1.2. Spectroscopic techniques.* It should be clear from the start that robust and reliable instrumentation that at minimum can be referenced and normalised to a manufacturer's standard should be used when developing methodologies that are to be used and transferred to others to perform non-targeted analysis using chemometric models. If spectra cannot be normalised in some form then it is impossible to deploy the methodology. When the methodology has been deployed, it is important that the sample preparation, if any, and the way in which to present the sample to the instrumentation is also clearly disseminated otherwise the integrity of the methodology will be compromised. Furthermore developers should be aware that different spectral responses across wavelength region is possible between different instruments in the same class. In terms of spectroscopy technology used in the 112 published articles, there is a fairly even split between FT-IR and NIR with 49 and 47 instances respectively. Raman was used on 31 occasions, Table 1. The total number of uses add up to more than 112 because in some articles more than one technology was applied. In terms of dairy there is a fairly even division in usage of technologies, with FT-IR, NIR and Raman seeing 11, 9 and 13 instances of technology use respectively. For meat there is a similar division between FT-IR (6) and NIR (8) with Raman being the technology used in 4 articles. FT-IR is the platform in 17 of the 22 articles for oil with NIR and Raman used 5 and 6 time respectively. All 6 Raman instances were used for olive oil applications, Table 1.

*2.3.1.3. Chemometric methods.* The chemometric technique chosen to facilitate the identification of food adulteration is independent of chosen technology platform. Quantitative chemometric techniques such as partial least squares regression and principal component regression have been excluded because the practical application of these techniques are more targeted in nature and seek to ascertain the quantity of a species. Classification models can be divided into unsupervised and supervised techniques. Additional information on classification models that can be applied to spectral data can be found in Marini, 2013. Unsupervised techniques include principal component analysis whilst supervised techniques such as soft independent modelling by class analogy and one-class partial least squares are truly non-targeted chemometric approaches because they can be used to create a cluster for spectra of known authentic product, which unknown samples can be compared and characterised against. If the test sample falls outside the cluster then it is a suspect sample that should undergo further evaluation using alternative analytical techniques. Bearing in mind the question being asked in non-targeted analysis is “is this product authentic?” or “is the sample from a particular group, e.g geographical origin?” and not seeking to quantify one particular adulterant. Other supervised chemometric techniques that assign to more than one class (e.g. partial least squares-discriminant analysis, linear discriminant analysis and k-nearest-neighbours) have been included as well. This is because not only can they show which class a sample belongs in, whether authentic class or known other, but they can also indicate that a sample does not belong to any of the classes, indicating a ratio between authentic commodity and adulterant or indeed a potential un-modelled

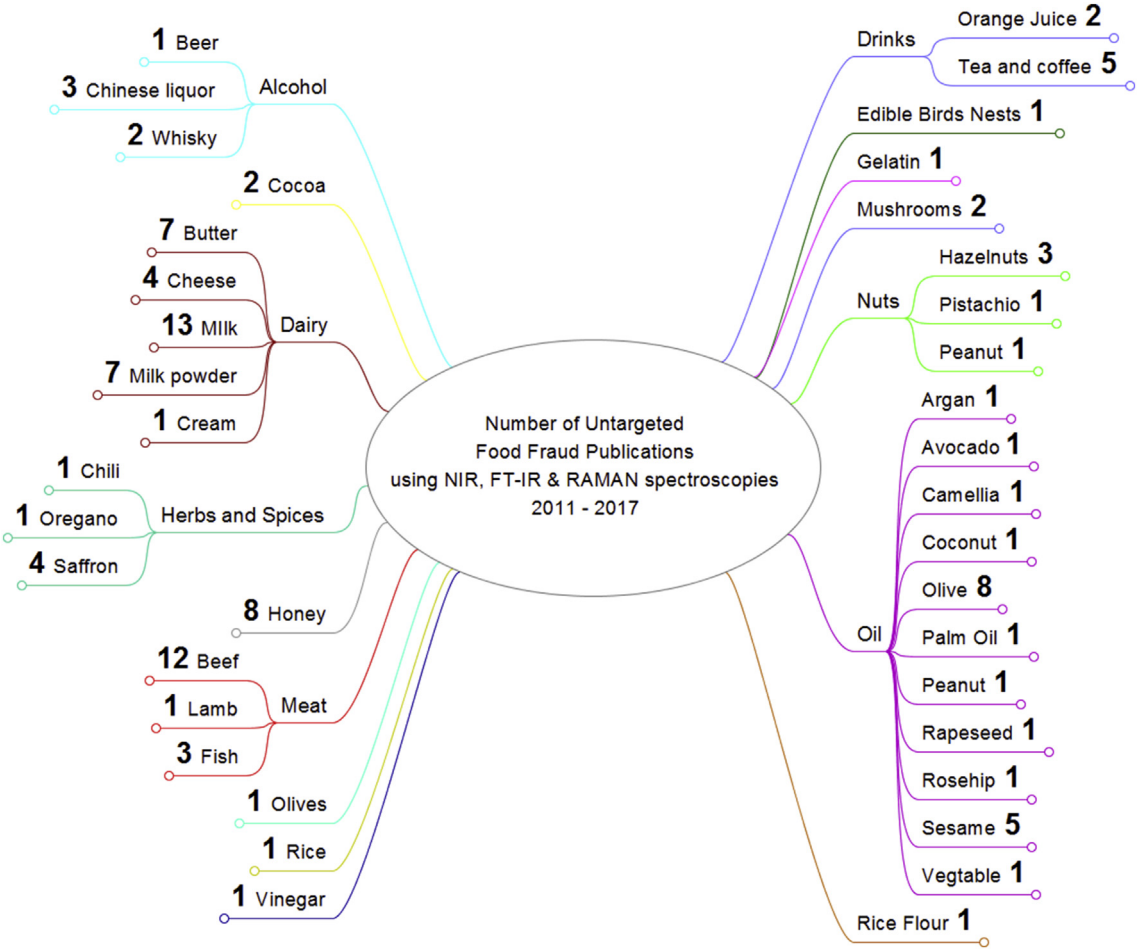


**Fig. 2.** Breakdown of scientific publications for optical spectroscopy techniques meeting the selection criteria.

adulterant or an un-modelled authentic product.

As expected, principal component analysis is the most common chemometric technique employed across the articles (79 instances of use), Table 1. This is unsurprising since this unsupervised technique is often used to look for natural clustering within samples before other supervised chemometric techniques are developed and employed. (Orthogonal) partial least squares-discriminant analysis was the most widely used supervised technique with 45 instances followed by soft independent modelling by class analogy and (linear) discriminant analysis (both on 27 instances), and support vector machine (14 instances) before the chosen chemometric technique drops to single digit use, Table 1.

### 2.3.2. Review of method development and validation for beef, milk and olive oil

One of the most substantial challenges to the adoption of non-targeted techniques is the lack of generally accepted standardisation governing the validation and implementation of non-targeted methods. In the meantime, in terms of applications, there still remains an important space for them to be used as rapid screening tools, whilst legislation is developed, with suspect samples subjected to some form of targeted/confirmatory analysis. However, guidelines remain absent which has resulted in various approaches being adopted in terms of sampling and method performance as can be evidenced in Table 2 (extended with data in Supplementary Table 2). It is clear that, as a starting point, guidelines are urgently needed which should ultimately result in legislation to standardise and harmonise the processes involved in method development and validation. To advance the discussions, we have sought to point out the variations observed in the articles that met the selection criteria. Due to the number of publications (112) identified, it was decided to focus on the three commodities most commonly researched (beef, milk and olive oil) using non-targeted vibrational spectroscopic approaches to detect food adulteration. Table 2 and Supplementary Table 2 provide an overview of data relating to key criteria that describe these publications. The following sections will evaluate these findings and provide both challenges for the analytical and regulatory communities as well as suggested recommendations to follow.

*2.3.2.1. Objective of methodology*. There are three common objectives covering all commodities and publications listed in Table 2 and Supplementary Table 2:

- Detection of adulteration – the presence of an undeclared substance
- Differentiation between commodity types e.g. discriminating between (or substitution of) buffalo and (with) cow milk
- Proving geographic origin e.g. identifying if a commodity is a Protected Designation of Origin (PDO) commodity

Product adulteration and differentiation can be closely related terms and in some instances have been used interchangeably. However these have been kept separate based on original article descriptions. An example of differentiation that is not necessarily a case of adulteration is the classification of extra virgin olive oil according to brand (McReynolds, Garcia, Guengerich, Smith, & Dholakia, 2016). Of the thirty-three articles, covering the thirty-five records in Table 2, the vast majority (27) address product adulteration whilst differentiation between commodities occurs five times and geographic origin on three

**Table 1**
Commodities, number of papers, and methodology employed in the articles that meet the selection criteria used for the literature search.

| Commodity | Sub-commodity | Number of papers | Method | | | Chemometric method | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FT-IR/ MIR | (FT-)NIR | (FT-) Raman | PCA | (O)PLS-DA | SIMCA | (L)DA | SVM | k-NN | OCPLS | ANN | (H)CA | UNEQ | (S)LLE | ECVA | QDA | Perceptron | GA | PLS-DM | n-CC | CLPP |
| Alcohol | Beer | 1 | 1 | | | 1 | | | 1 | | 1 | | | | | | 1 | 1 | | | | | |
| | Chinese Liquor | 3 | | 3 | | 2 | 1 | | 1 | 2 | 1 | | | | | | | | 1 | | | | |
| | Whiskey | 2 | 1 | | 1 | 2 | | | | | | | | | | | | | | | | | |
| Cocoa | | 2 | | 2 | | 2 | | | 1 | 2 | 1 | | 1 | | | | | | | | | | |
| Dairy | Butter | 7 | 4 | 1 | 2 | 3 | 1 | 1 | 2 | | | | | | | | | | | | | | |
| | Cheese | 4 | 1 | 2 | 1 | 2 | 2 | | 1 | | | | 1 | | | | | | | | | | |
| | Milk & milk powder | 20 | 6 | 6 | 9 | 12 | 8 | 6 | | 2 | 1 | 2 | 1 | | | | | | | | | | |
| | Cream | 1 | | | 1 | 1 | | | 1 | | | | | | | | | | | | | | |
| Drinks | orange Juice | 2 | 2 | | | 3 | | | 1 | | | | | | | | | | | | | | |
| | Tea & Coffee | 5 | 3 | 3 | | 3 | 1 | 1 | 1 | 1 | | 1 | | 1 | | | | | | | | | |
| Edible Birds Nests | | 1 | | 1 | | 1 | | | | | 1 | | | | | | | | | 1 | | | |
| Gelatine | | 1 | 1 | | | 1 | | | | | | | | 1 | | | | | | | | | |
| Herbs & Spices | Chili | 1 | | 1 | 1 | 2 | 2 | | | | | | | | | | | | | | | | |
| | Oregano | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | | |
| | Saffron | 4 | 3 | | 1 | 3 | 1 | | 1 | | | | | | | | | | | | | | |
| Honey | | 8 | 3 | 5 | 3 | 5 | 6 | 1 | 1 | 1 | 1 | | 1 | 1 | | | | | | | | | |
| Meat | Beef | 12 | 5 | 5 | 3 | 10 | 6 | 4 | 4 | 2 | | | | | | | | | | | | | |
| | Lamb | 1 | | 1 | | 1 | 1 | | 1 | | | | | | | | | | | | | | |
| | Fish | 3 | 1 | 2 | 1 | 3 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| Mushrooms | | 2 | | 2 | | 1 | | 1 | | 1 | | 1 | | | 1 | | | | | | 1 | | |
| Nuts | Hazelnuts | 3 | | 3 | 1 | 2 | 1 | 3 | | 1 | 1 | | | | | | | | | | | | |
| | Pistachio | 1 | | 1 | | | 1 | 1 | | | | | | | | | | | | | | | |
| | Peanut | 1 | | 1 | | 1 | 1 | | | | | | | | | | | | | | | | |
| Oil | Argan | 1 | 1 | | | 1 | 1 | | | | | | | | | | | | | | | | |
| | Avocado | 1 | 1 | | | | | 1 | | | | | | | | | | | | | | | |
| | Camellia | 1 | | 1 | | 1 | | | 1 | | | | 1 | 1 | | | | | | | | | |
| | Coconut | 1 | 1 | | | | | | 1 | | | | | | | | | | | | | | |
| | Olive | 8 | 5 | 3 | 6 | 7 | 2 | 1 | 3 | 1 | 1 | | | | | 2 | | | | | | 1 | 1 |
| | Palm | 1 | | 1 | | 1 | | 1 | | | | | | | | | | | | | | | |
| | Peanut | 1 | 1 | | | 1 | 1 | 1 | | | | | | | | | | | | | | | |
| | Rapeseed | 1 | 1 | | | | | | 1 | | | | | | | | | | | | | | |
| | Rosehip | 1 | 1 | | | | 1 | | | | | | | | | | | | | | | | |
| | Sesame | 5 | 5 | | | 4 | 4 | 2 | 3 | | | | | | | | | | | | | | |
| | Vegetable | 1 | 1 | | | | | | 1 | | | | | 1 | | | | | | | | | |
| Olives | | 1 | | 1 | | 1 | | | | | | | | | 1 | | | | | | | | |
| Rice | | 1 | | | 1 | 1 | 1 | 1 | | 1 | 1 | | | | | | | | | | | | |
| Riceflour | | 1 | | 1 | | | | 1 | | | | 1 | | | | | | | | | | | |
| Vinegar | | 1 | | 1 | | | 1 | | | | | | | | | | | | | | | | |
| SUM | | 112 | 49 | 47 | 31 | 79 | 45 | 27 | 27 | 14 | 9 | 5 | 5 | 5 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

PCA: Principal component analysis, (O)PLS-DA: (orthognol) partial least squares discriminant analysis, SIMCA: Soft independent modelling by class analogy, (L)DA: (Linear) discriminant analysis, SVM: Support Vector Machine, k-NN: k-Nearest-Neighbours, OCPLS: one-class partial least squares, ANN: artificial neural networks, (H)CA: (hierarchical) cluster analysis, UNEQ: unequal dispersed classes, ((S)LLE): (Supervised) locally linear embedding, ECVA: extended canonical variates analysis, QDA: quadratic discriminant analysis, GA: genetic algorithm, PLS-DM: partial least squares density modelling, n-CC: Nearest centroid classification, CLPP: Continuous Locality Preserving Projections.

**Table 2**
Summary of main findings following review of articles, which meet the selection criteria, concerning food fraud related to beef, milk and olive oil. Further details presented in Supplementary Table 2.

| Commodity | Adulterant investigated | Sample source | Total No Samples | Spectroscopy | Software | Data processing | Chemometric methods | Reference samples | Test samples | validation set | Performance | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Beef (minced) | Turkey | beef from producer, turkey breast local supermarket | 242 | FT-NIR | Matlab | SNV; first derivative | PCA; LDA | ? | ? | 66 | PCA: 2PCs 98% variance - couldn't distinguish low level contamination (< 20%); LDA: correctly classified average 71.2–88.3% | (Alamprese, Casale, Sinelli, Lanteri, & Casiraghi, 2013) |
| Beef (minced) | Turkey | | 242 | FT-IR (ATR) | Matlab | SNV; S-G smoothing (first derivative) | PCA; LDA | ? | ? | 66 | PCA: 2PCs 82.3% variance - couldn't distinguish low level contamination(< 20%); LDA: correctly classified average 65.2–84.8% | (Alamprese et al., 2013) |
| Beef (minced) | Pork; fat; offal | raw materials from Retail outlets | 191 | VIS-NIR | ? | Moving average and S-G smoothing (second derivative); MSC; SNV | LDA; PLS-DA | 126 | 65 | No | LDA & PLS-DA 100% correctly classified adulteration in fresh meat, 78–100% correctly classified in thawed meat | (Morsy & Sun, 2013) |
| Beef burgers | Beef offal | raw materials from Retail outlets | 82 | NIR | Unscrambler | MSC; SNV; S-G smoothing (first and second derivative) | PCA; PLS-DA; SIMCA | PLS-DA 41; SIMCA 18 | PLS-DA 41; SIMCA 59 | No | PCA: 2PCs 99% variance; PLS-DA: 88.9–100% correctly classified depending on fresh/frozen; SIMCA: **Sensitivity** 0.88–1, **Specificity**: 0.99–1, efficiency 0.94–1 | (M. Zhao et al., 2013) |
| Beef | Horsemeat | Beef from supermarkets, horsemeat from markets | 49 | Raman | version solo | baseline correction; derivatives; MC; normalizing; smoothing; auto-scaling | PCA | 39 | 10 | No | PCA: 2PCs 99.5% variance, separate horsemeat from beef. Binary mixtures ranging 25%–75% show own clusters between both. Successfully classified 25% adulteration | (Boyaci et al., 2014) |
| Meat and salami products | Fat from: cattle; sheep; pig; fish; poultry; goat; buffalo | supermarkets and slaughter houses | 111 meat +21 salami | Raman | version solo | baseline correction; derivatives; MC; normalizing; smoothing; auto-scaling | PCA | 111 meat | 21 salami | No | PCA: multiple models model 1: 2 PCs 86.84% variance, produced 4 clusters; model 2: 2PCs 91.5% variance, could separate goat from within its mixed cluster; model 3 2PCs 99% variance, split cattle, sheep and goats from mixed cluster; model 4 separated pig and buffalo from mixed cluster. Could distinguish unary salami but additional processing needed to classify binary samples | (Boyaci et al., 2014) |
| Beef burgers | Beef offal | raw materials from Retail outlets | 82 | FT-IR (ATR) | Unscrambler | MC; MSC; SNV; S-G smoothing (first and second derivative) | PCA; PLS-DA; SIMCA | 41 | 41 | No | PCA: 3 PCs 92.6% variance; couldn't distinguish fresh from thawed, discernible pattern between adulterated and authentic in fresh; PLS-DA: 97.2–100% correctly classified; SIMCA: **Sensitivity**: 0.94–1; **Specificity**: 0.33–0.87; efficiency: 0.57–0.91 | (M. Zhao et al., 2014) |
| Beef meatballs (cooked) | Pork | Slaughter houses for raw materials | 23 | NIR | Unscrambler | first and second derivative | LDA | 11 | 5 | 7 | LDA: 80–100% correctly classified | (Kuswandi, Cendekiawan, Kristiningrum, & Ahmad, 2015) |

(*continued on next page*)

**Table 2** (*continued*)

| Commodity | Adulterant investigated | Sample source | Total No Samples | Spectroscopy | Software | Data processing | Chemometric methods | Reference samples | Test samples | validation set | Performance | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Beef jerky | Pork | beef and pork from slaughter houses. Other materials local market | 43 | FT-IR (ATR) | Unscrambler | normalised; baseline corrected and smoothed | LDA; SIMCA; SVM | 20 | 5 | 18 | SVM: correctly classified 80–100%, Prediction 80%; SIMCA: correctly classified 100%, Prediction 0–60%; LDA: correctly classified 100%, Prediction 80–100%, 10 of the validation set (labelled halal) tested by LDA and Elisa with 100% agreement - doesn't say about the other 8 | (Kuswandi et al., 2015) |
| Beef meatballs | Rat | rat meat from farms, beef from markets | 11 | FT-IR (ATR) | Horizon MB | ? | PCA | 7 | No | 4 | PCA: distinguish 100% rat meat from 100% beef, 2 validation samples similar to beef 2 similar to rat (or other formulation) | (Rahmania, Sudjadi & Rohman, 2015) |
| Beef burgers | Beef offal | raw materials from Retail outlets | 81 | Raman | Unscrambler | normalization to unit vector length (u.v.nor); S-G smoothing (first and second derivative) | PCA; PLS-DA; SIMCA | 41 | 40 | No | PLS-DA: correctly classified 89–100%; SIMCA: **Sensitivity**: 0.94–1, **Specificity**: 0.64–1, efficiency:0.80–0.97 | (M. Zhao et al., 2015) |
| Beef (minced -fresh, frozen-thawed, cooked) | Turkey | ? | 198 | FT-NIR | Matlab | SNV; MSC; S-G smoothing (first and second derivative) | PCA; PLS-DA | 144 | 54 | No | PCA: gradient distribution based on % adulteration. Pure turkey well separated from beef (0–50% adulteration); PLS-DA: 20% adulteration threshold. All: **Sensitivity**: > 0.84, **Specificity**: > 0.76 | (Alamprese et al., 2016) |
| Beef | NaCl; phosphates; carrageenan; maltodextrin | police raids | 55 | FT-IR (ATR) | Matlab | class centroid centering; S-G smoothing; MSC | PCA; PLS-DA | 38 | 17 | No | PCA: 3PCs 94.2% variance. No clear discrimination; PLS-DA: 16.7–23.1% false negative, 25–37.5% false positive, **Sensitivity**: 76.9–83.3%, **Specificity**: 51.9–62.5%, efficiency 45.8–51.9%, AUROC 0.75 - however better results on data fusion | (Nunes, Andrade, Santos Filho, Lasmar, & Sena, 2016) |
| Milk | sodium bicarbonate; sodium citrate; non-acid cheese whey | farms | 900 | FT-IR | ASM | ? | PCA | 800 | 100 | No | Depends on number of PCs used; Could 100% distinguish bicarbonate at 0.05% w/v and 0.075% w/v citrate > 93% but poor results for Whey at any concentration, **Sensitivity**: 87.9–92.9% | (Cassoli et al., 2011) |
| Milk | Protected Designation of Origin | Farms | 486 | NIR | ? | SNV; detrend scatter correction | PLS-DA | 419 - 473 depending on question asked | 13-67 depending on question asked | No | Cross validation error 3.6–37.8% depending on question asked, external validation error 3.8–11.2% depending on question asked | (Coppa et al., 2012) |
| Milk | Whey; hydrogen peroxide; synthetic urine; urea; synthetic milk | Retail | 370 | FT-IR (ATR) | Pirouette | Normalised; S-G smoothing (second derivative); MC | SIMCA | 196 | 174 | No | SIMCA: allowing separation of adulterated from non-adulterated. Correctly classified 90–98%. | (Santos et al., 2013) |
| Milk | | | 813 | FT-IR | Matlab | SNV | | 542 | 271 | No | | |

(*continued on next page*)

**Table 2** (*continued*)

| Commodity | Adulterant investigated | Sample source | Total No Samples | Spectroscopy | Software | Data processing | Chemometric methods | Reference samples | Test samples | validation set | Performance | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Melamine; urea; ammonium nitrate | Dairy Research Institute | | | | | PCA; improved and simplified-kNN; improved-SVM | | | | PCA: 20PCs 99.9996% variance. I-SVM: validation correct ratio-false: > 90%, validation correct ratio-True: > 86%; IS-kNN: validation correct ratio-false: 90%, validation correct ratio-True: > 86% | (Zhang et al., 2014) |
| Milk | Water; starch; Na citrate; formaldehyde; sucrose | Veterinary School of the Universidade Federal de Minas Gerais | 193 | FT-IR (ATR) | Matlab | S-G smoothing (first derivative); MC | PLS-DA | 155 | 38 | No | False positives: 0–11.5%; False negatives: 0–16.7% **Sensitivity**: 83.3–100%; **Specificity**: 88.5–100%; efficiency: > 75.3–100% for all 5 adulterants covering training and test sets | (Botelho, Reis, Oliveira, & Sena, 2015) |
| Milk | Urea | Farm | 210 | FT-IR (ATR) | Unscrambler | wavelength selection | PCA; SIMCA | 105 | 105 | No | PCA: 2PCs 100% variance, Identified 3 groups: milk, milk with urea < 900 ppm, milk with urea > 900 ppm. SIMCA: also 3 clusters. correctly classified > 80% although no false negatives | (Jha, Jaiswal, Borah, Gautam, & Srivastava, 2015) |
| Milk (UTH) | Water; urea; formaldehyde | supermarkets | 240 | FT-NIR | Matlab | MC; MSC; OSC; S-G (first and second derivative) | PLS-DA; SIMCA; SMV-DA | 120 | 117 | No | SIMCA: between 60 and 100% discrimination; SVM-DA: between 80 and 100% discrimination; PLS-DA: between 91 and 100% discrimination based on adulterant; All: **Sensitivity**: 48–100%, **Specificity**: 43–100% | (Luna et al., 2016) |
| Milk | melamine, urea, ammonium sulfate, dicyandiamide, sucrose | Retail | 64 | Raman | Unscrambler | S-G smoothing (second derivative) | PCA; PLS-DA | 40 | 24 | No | PCA: could not distinguish unadulterated from adulterated with concentrations below 500 mg/L; PLS-DA: predicted 100% adulterated samples, selectivity rate 100%, false negative rate 0%. 2 false positives (rate of 15.4%), efficiency 84.6% | (Nieuwoudt et al., 2016a) |
| Milk | melamine, urea, dicyandiamide, ammonium sulfate, sucrose | Retail | 40 | Raman | Unscrambler | S-G smoothing (derivative function); baseline levelling; MC | PLS-DA | 20 | 20 | No | PLS-DA: **Sensitivity**: 100%, **Specificity**: 100% | (Nieuwoudt et al., 2016b) |
| Milk | Cow: buffalo | Farms | 30 | Raman | Matlab | Mean normalizing; S-G smoothing | PCA | No | No | No | could distinguish between cow and buffalo | (Ullah et al., 2017) |
| Milk | Formaldehyde; hydrogen peroxide; bicarbonate; carbonate; chloride; citrate; hydroxide; hypochlorite; starch; sucrose; water | Experimental Farm | 360 | FT-IR (ATR) | Matlab | MSC; MC | SIMCA | Leave one out cross validation | | No | SIMCA: one class model: **Sensitivity**: 93.1; **Specificity**: 56–100%; Reduced multi class model: **Sensitivity**: 70–93.1%, **Specificity**: 93.3–100% | (Gondim et al., 2017) |

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**Table 2** (*continued*)

| Commodity | Adulterant investigated | Sample source | Total No Samples | Spectroscopy | Software | Data processing | Chemometric methods | Reference samples | Test samples | validation set | Performance | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Milk | Detergent | Supermarkets | 11 | FT-IR(ATR) | Unscrambler | wavelength selection | PCA; SIMCA | 11 (split replications over ref and Test) | | No | PCA: 2PCs 99% variance. Clearly discriminate pure from adulterated (4 clusters); SIMCA: 4 clusters efficiency: > 93%, LOD 0.2% adulterated | (Jaiswal et al., 2017) |
| Milk | Melamine; ammonium sulphate; dicyandiamide; urea; sucrose | ? | 40 | Raman | Unscrambler | S-G smoothing (derivatisation); MC | PLS-DA | 20 | 20 | No | PLS-DA: **Sensitivity**: 92%, **Specificity**: 89% | (Nieuwoudt et al., 2017) |
| VOO | Protected Designation of Origin | Olive oil association | 396 | Raman | Unscrambler | SNV; MSC | PLS-DA | 266 | 130 | No | PLS-DA: correct PDO prediction between 80 and 100% depending on region | (Korifi, Le Dreau, Molinet, Artaud, & Dupuy, 2011) |
| EVOO | Corn oil; sunflower oil | Supermarkets | 40 | FT-IR(ATR) | TQ- Analyst | ? | DA | ? | ? | No | DA: correctly classified EVOO from adulterated EVOO, 100% classification | (Rohman & Man, 2012) |
| EVOO | Camellia; soybean; sunflower; corn oils | Supermarkets | 300 | FT-IR(ATR) | Matlab | MC; normalization; S-G smoothing (first derivative) | PCA; LLE; nearest centroid; SLLE | 183 | 117 | No | PCA: 3PCs 99% variance, could not distinguish between samples; LLE: better distinguish than PCA; SLLE: better distinguish than PCA; SLLE + nearest centroid: best results misclassification rate training set 1% misclassification rate test set 3.4% | (Sun et al., 2015) |
| VOO | Different grades | Producers | 70 | FT-IR | Unscrambler | S-G smoothing (first derivative) | PLS-DA | 50 | 20 | No | PLS-DA: 4 clusters, R-squared in calibration: 0296–0.98, root mean squared error in calibration 0.04–0.08, Root mean squared prediction error in external validation: 0.1–0.16. 100% correct classification of validation set | (Hirri, Bassbasi, Platikanov, Tauler, & Oussama, 2016) |
| EVOO | Various vegetable oils | Producers and supermarkets | 193 | FT-NIR | Matlab | S-G smoothing (first derivative); SNV | PCA; SIMCA | 55 | 37 | 101 | PCA: 6PCs 99% variance; SIMCA: 100% correctly classified test samples. **Sensitivity**: 100%, **Specificity**: 100%, Validation samples: all samples labelled blends correctly classified not EVOO, 5 EVOO ref samples wrong classification. Only 34 of 88 labelled EVOO commercial samples classified EVOO (possible quality issues or limited no oils used for reference) | (Karunathilaka, Farris, Mossoba, Moore, & Yakes, 2017) |
| EVOO | Different brands | Supermarkets | 5 | Raman | Matlab | Normalized (area under the curve) | PCA | Leave one out cross validation | | | PCA: well defined clusters for the 5 brands. **Sensitivity**: 98.4%, **Specificity**: 99.6% | (McReynolds et al., 2016) |
| EVOO | | Co-op | | FT-Raman | ? | S-G smoothing | LDA | | | | | (Sanchez-Lopez et al., 2016) |

(*continued on next page*)

**Table 2** (*continued*)

| Commodity | Adulterant investigated | Sample source | Total No Samples | Spectroscopy | Software | Data processing | Chemometric methods | Reference samples | Test samples | validation set | Performance | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Year; variety; Protected Designation of Origin | | 412/ 307/ 145/67 | | | | | Leave one out cross validation | | | LDA: Harvest year 94% correctly classified; Olive variety: 84% correctly classified; Geo Origin: 89% correctly classified; PDO: 86.6%correctly classified | |
| EVOO | Hazelnut oil | Producers | 264 | FT-IR (ATR) | Matlab | SNV; S-G smoothing | PCA; CLPP; kNN; LDA; Pearson's Correlation; PLS-DA; SIMCA; SVM; UHC | 168 | 18 | No | PCA: no clear separation of admixtures; LDA: better separation; CLPP: good visual separation; Overall classification rate 56–75% depending on method. For adulteration < 12% classification rate between 27 and 70%. Best results obtained using CLPP + kNN | (Georgouli, Del Rincon, & Koidis, 2017) |
| EVOO | Hazelnut oil | Producers | 264 | Raman | Matlab | SNV; S-G smoothing | PCA; CLPP; kNN; LDA; Pearson's Correlation; PLS-DA; SIMCA; SVM; UHC | 168 | 18 | No | PCA: no clear separation of admixtures; LDA: better separation; CLPP: good visual separation; Overall classification rate 53–79% depending on method. For adulteration < 12% classification rate between 12.5 and 82%. Best results obtained using CLPP + kNN | (Georgouli et al., 2017) |

MC: mean centering; MSC: Multiplicative Scatter Correction; OSC: Orthogonal signal correction; S-G: Savitzky-Golay; SNV: Standard Normal Variate; CLPP: Continuous Locality Preserving Projections; DA: Discriminant Analysis; kNN: K Nearest Neighbours; LDA: Linear Discriminant Analysis; PCA: Principal Component Analysis; PLS-DA: Partial Least Squares Discriminate Analysis; SIMCA: Soft Independent Modelling of Class Analogy; (S)LLE: (Supervised) Locally Linear Embedding; SVM: Support Vector Machine; UHC: Unsupervised Hierarchical Clustering; (E)VOO: (Extra) Virgin Olive Oil.

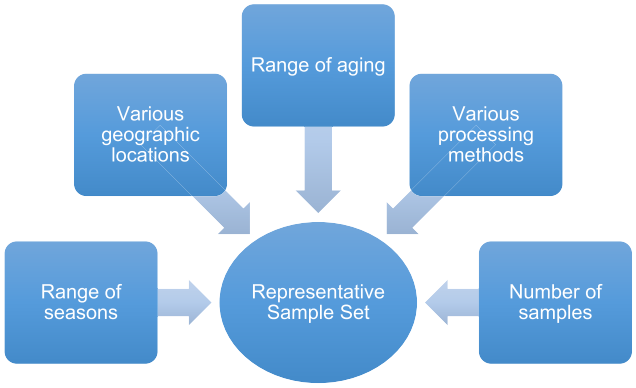


**Fig. 3.** Elements to consider when developing a representative sample set.

occasions.

Of the thirteen records for beef, all but one (differentiation of unary salami products based on source of meat (Boyaci, Uysal et al., 2014)) relates to adulteration. Differentiation and geographic origin represent two records for milk with the other 11 address product adulteration. Differentiation appears to be of most interest in olive oil with four records, followed by adulteration with three records, and geographic origin with one record.

*2.3.2.2. Adulterant investigated*. As expected, almost all beef related articles focus on the inclusion of other species of meat (including horse, turkey, pork and rat) or offal, whilst milk articles cover a broad range of adulterants including nitrogen based compounds, stabilisers, preservatives and detergents. For extra virgin olive oil the main concern remains the addition of cheaper oils, Table 2.

*2.3.2.3. Sample related criteria*. Fig. 3 presents the elements to consider when developing a representative samples set.

*2.3.2.3.1. Sample source*. Two of the records in Table 2 have not clearly identified the sample source (Alamprese, Amigo, Casiraghi, & Engelsen, 2016; Nieuwoudt, Holroyd, McGoverin, Simpson, & Williams, 2017), while fourteen records indicate that samples were, at least in part, obtained from supermarkets and other retail outlets (Boyaci, Temiz et al., 2014; Boyaci et al., 2014; Jaiswal, Jha, Kaur, & Borah, 2017; Luna, Pinho, & Machado, 2016; McReynolds et al., 2016; Morsy & Sun, 2013; Nieuwoudt, Holroyd, McGoverin, Simpson, & Williams, 2016a; Nieuwoudt, Holroyd, McGoverin, Simpson, & Williams, 2016b; Rohman & Man, 2012; Santos, Pereira-Filho, & Rodriguez-Saona, 2013; Sun, Lin, Li, Shen, & Luo, 2015; M.; Zhao, O'Donnell, & Downey, 2013; M.; Zhao, Downey, & O'Donnell, 2014; M.; Zhao, Downey, & O'Donnell, 2015). The remainder of the publications had samples collected from slaughter houses, producers, farms or accreditation bodies. Thus we can argue that approximately 48% of the publications did not have suitably authenticated samples and the resultant data is of limited value. Sourcing of samples is critical for model development for non-targeted methods using spectroscopy. The samples must be authentic and of known and provable provenance, ideally with full traceability. Samples should be obtained from reputable sources preferentially directly from the producer of the particular commodity at the position within the supply chain where the methodology is to intervene. It is unwise to collect samples from retail outlets or markets for model development since these sources are at the end of the supply chain and the material will have passed through many hands.

Despite this goal of very high standards in terms of sample collection, we acknowledge that collecting authentic samples is extremely challenging and requires collaboration with the food industry. In some cases it may also be possible that if a sufficient number of samples are collected then unsupervised techniques such as PCA-class analysis could identify suspect samples requiring exclusion or further investigation. An alternative source of authentic samples for model development could be the use of a wide range of certified reference materials. However this is a challenge to the community in that there is an extreme lack of such samples for foods. Potential sources to look for certified reference materials include the European Commission Joint Research Centre or through distributors such as Sigma-Aldrich.

*2.3.2.3.2. Number of samples*. Table 2 outlines the wide range in the total number of samples used for both development and validation of non-targeted spectroscopy methodologies. These range from as low as five (McReynolds et al., 2016) to as high as high as 900 (Cassoli, Sartori, Zampar, & Machado, 2011). The median number of total samples used is 168 (mean 201 samples) The majority of publications (23) use less than 200 samples of which 17 use less than 90 samples with only nine publications using more than 300 samples in total.

When developing non-targeted methodologies, it is extremely important to collect a sufficient number of unique samples to cover variations associated with the commodities of interest (both in terms of the chosen commodity and suspected adulterants). In order to generate a robust model this includes variables such as season, storage, natural commodity variations, location etc. Initial models developed may not offer the robustness needed for long term usage but they can be added to over time as new season commodities become available. The authors' opinion is that the majority of published articles reviewed have used sufficient samples to provide a limited proof-of-concept for their method but have not generated sufficiently robust models to employ the method as a tool for regulatory purposes. The rationale for this conclusion is as follows: if the median number of samples is 168 and best case scenario equal numbers of unique samples have been collected for the authentic commodity and only one potential adulterant that would mean that each population would consist of 84 samples only. This very low number brings into question the required diversity in sample types needed to build robust models. Furthermore the majority of papers describe a 2/3 split in samples between Reference samples (to build the model) and Test samples (to evaluate the model performance) meaning that on average, only 112 samples in total were employed to build the model. Therefore this could be as low as only 56 samples per class in a 2 class model. It is difficult to believe that a robust model, covering all natural variations could be developed with such limited sample numbers. The recommendation of the authors of this Scientific Opinion is that at least a minimum of 200 representative samples should be collected for each class in the model. This is based on a review of empirical data from our own experiences whereby model performances have undergone evaluation through the various iterations of their maturity. Findings show that early models with lower sample numbers generally do not sufficiently represent the normal sample population. As the reference sample set is increased the models robustness improves. In order to represent normal sample distribution, both historical and current, models need to be kept "live" and updated to ensure their robustness for future analysis needs.

*2.3.2.3.3. Representative samples*. The total sample number is an even more complicated story than the simple explanation described above. In some instances the total sample data in Table 2 is misleading in terms of representative samples. This is because some articles describe each spiked sample as a unique sample. For example Gondim et al. (Gondim, Junqueira, Carvalho de Souza, Ruisanchez, & Pilar Callao, 2017) and Santos et al. (Santos et al., 2013). Gondim et al. describe analysing 360 milk samples. However they only collected 30 samples from the farm and produced spiked samples for 11 potential adulterants at various concentrations whereas Santos et al. describe the analysis of 370 samples but collect retail sample, of the same brand of milk, from 10 different lot numbers to generate their 310 adulterated samples. From the perspective of variation in adulterant concentration they both have a good range but in real terms of representative samples of milk they have only included 30 or 10 unique samples. It is highly unlikely that these spectra will represent all the variation expected in

natural milk samples collected from different animals on different farms at various milking times over numerous seasons. A further recommendation from the authors' relates to using spiked samples i.e. the total number of samples stated should represent the samples collected and not the samples created through spiking.

*2.3.2.3.4. Use of spiked samples*. Spiked samples have been used in all of the publications analysed where the objective of the method is to detect adulteration (Supplementary Table 2). This is expected when samples of known concentration are required to build models. However, it is important to consider how the spiked samples were prepared and if this matches the real world industrial process that real commodities undergo. Failure to follow such processing procedures may mean that a laboratory validated method will not be transferrable to industrial products because the prepared spiked samples do not represent real processed commodities. For example the use of laboratory grinders to prepare shop bought fillets for burger preparation: has the same quality meat cuts been used? Does the grinding process mirror the industrial scale process? Have all the additional typical industrial scale processing steps been included e.g. inclusion of preservatives etc.? In order to make the method transferrable it is imperative to at least understand and attempt to match that industrial process as thoroughly as possible.

The draft USP guidance calls for spiked samples to be prepared at three levels (at a concentration around a risk threshold, half that concentration and double that concentration). The idea of three spiked concentrations in general has been followed by all publications outlined in Table 2 (Supplementary Table 2). However, this poses a challenge in that no official guidance is available on what concentration samples should be spiked at. The authors' recommend that a common sense, intelligence led approach is adopted when defining the initial concentration of interest. Not only should the alleged adulterant be intelligence led but the concentration at which it is likely to be used at e.g. the minimum concentration that makes economic sense to the fraudster.

The number of spiked samples prepared for each adulterant is also important. The draft USP guidance suggested at least three. From a robustness perspective this seems low especially if all spiked samples are prepared in the same sample batch. The authors' recommend that cross-representative samples of authentic samples are used to spike samples to account for natural variation within the sample.

It is also a recommendation within the draft USP guidance that spiked samples of mixed adulterants are not used and in the majority of publications, in Table 2 this is the case, Supplementary Table 2. However the authors suggest flexibility on this. The preparation of spiked samples should be intelligence led and if the use of multiple adulterants e.g. mixed off cuts, mixed offal is suspected in the real world then the modelling scenario must reflect this.

### *2.3.2.4. Instrumentation methodology*

*2.3.2.4.1. Spectroscopy technique analytical parameters*. As outlined, three spectroscopy techniques have been used for non-targeted analysis of beef, milk and olive oil and this section focuses on the analytical parameters used. Twenty six articles, in Supplementary Table 2, describe the number of replicates measured per sample of which 20 use a maximum of 3 replicates with only two using more than 10 replicates. Of the 23 publications that describe scans per sample, 10 use 32 scans per sample 6 use 24 scans per sample or less and only 3 using more than 120 scans per sample, Supplementary Table 2. The Raman applications that describe scanning time range from short 3 s scans to 240 s scans with the majority of articles (4) describing the use of 30 s scans. The number of scans used, or time, will be instrument dependent, commodity dependent and will reflect whether the developer has tried to improve the signal to noise ratio.

*2.3.2.5. Chemometric methodology*. Many users, as well as legislature, view chemometrics as a "Black Box" where there is limited understanding of what is happening "under the lid." This in and of itself presents a challenge that needs to be addressed to build confidence and understanding that will in turn lead to harmonized model development and wider acceptance of the methodology.

*2.3.2.5.1. Software used for chemometric analysis*. Of the 32 records providing details on software used for chemometric modelling 26 used either a version of Matlab, from MathWorks, (14) or a version of Unscrambler, from Camo, (12). Other software such as TQ Analyst, from Thermofisher, Pirouette, from Infometrix, R, from the Free Software Foundation and SIMCA, from Sartorius Stedim Biotech, are also widely used although not represented well in the commodities in Table 2. The lack of a single software solution that offers all algorithms, in a user friendly manner, in one package is a challenge to the analytical community. Matlab in conjunction with PLS Toolbox does provide a large range of algorithms however this may be cost prohibitive in non-academic situations. In that case developers will either purchase multiple licences covering additional software options or more likely make use of one package (set of algorithms) that is supported by their institute, at the expense of increased options for algorithms.

*2.3.2.5.2. Data processing*. Table 2 shows that a wide range of data processing steps have been applied in an iterative fashion by the various research groups. It is clear that there is no set formula to select which steps to perform and in what order, if at all (several articles describe models developed using raw spectra where the only processing has been wavelength selection). It is clear that data processing should be investigated in a scientific manner, based on the spectra from the commodity and the returned model performance characteristics.

*2.3.2.5.3. Chemometric models*. Likewise there can be no consensus on which chemometric model to apply to which commodity, other than suggesting developers investigate as many models as possible and select the one that gives the most favourable performance statistics, including empirical data from known samples, for that chosen commodity. PCA is used widely as an unsupervised technique to undertake a preliminary analysis of the data but several articles seek to take PCA analysis further and show its ability to cluster samples according to their adulteration status.

*2.3.2.5.4. Reference sample set size, test sample set size*. The USP draft guidelines has the following definitions:

- The Reference sample set is a population of representative authentic Typical (unadulterated) samples and data acquisition conditions
- The Test sample set which is made up of an approximately equal mix of Typical (unadulterated) samples (which must not form part of the Reference set), and Atypical (adulterated) samples. Atypical sample can be made by spiking Typical samples. The test set is used to challenge the model for optimisation

The reference set is used to create the models. The test set is used to challenge the model for optimisation.

Not all articles have published their ratio between Reference set and Test set but of those that have the ratio goes from as low as 0.3:1 (for one-class SIMCA models) to as high as 32:1 for multiclass models. Many articles, including those beyond the selection criteria of this Scientific Opinion, recommend an approximate 2:1 relationship when using more than a one class classification model e.g. SIMCA. Analysing the ratio for the beef, milk and olive oil applications, the majority of articles (11) describe an approximate 1:1 ratio with 7 describing a 2:1 ratio. A 4:1 ratio is the second most commonly applied split (9 articles). With 5 describing a 7:1 ratio or greater. However the issue of the split is further complicated by in some instances, the relatively small number of samples used.

Another concept study encouraged to use test sets that are also representative for the population, both of the authentic and non-authentic samples, but that it should capture additional variation where possible (Alewijn et al., 2016). This would yield a more robust model than spiking only.

*2.3.2.6. Model performance criteria.* As there are no official guidelines on non-targeted method development and as such there are no set parameters to use to assess the performance. The draft USP guidelines suggest the determination of:

- Sensitivity rate - the number of correct Atypical predictions from the method divided by the total number of true Atypical samples
- Specificity rate - the number of correct Typical predictions from the method divided by the total number of true Typical samples
- The use of Receiver operating characteristic (ROC) curves – where the area under the curve (AUC) is an indication of performance. A good non-targeted method should have an AUC close to 1

Assessing the types of performance criteria described in Table 2 it can be observed that thirteen out of thirty-five referred directly to sensitivity and specificity performance for at least one of the models developed. There articles tended to also include an efficiency parameter. Only two referred to using ROC curves, Supplementary Table 2.

Throughout the articles investigated, descriptives such as “could not distinguish”, “produced clusters” etc. were used as general performance characteristics but in terms of quantifiable parameters the most commonly used term related to the correct classified rate or similar terms which in general told how well the model was able to assign a known sample into the correct class. Other terms used included: prediction ability; false negative/positive rate; validation error; misclassification rate.

The draft USP guidelines call for the Test set to have approximately an equal mix between Typical and Atypical samples. This is only the case in five of the thirty five records studied, Supplementary Table 2. Furthermore there is a suggestion that results are confirmed by a reference or secondary method. In many instances the publications indicate the use of spiked samples with known concentrations and thus no secondary testing was performed, Supplementary Table 2.

One excellent way to test the performance of a model is the use of reference materials. However such materials must not have been used in the model creation. Only one of the papers mention the use of a reference material at some point in the development/validation of the non-targeted method, Supplementary Table 2.

Another option would be to test the model results by performing a ring trial however there was no indication in any of the publications that this was undertaken, Supplementary Table 2. In the real world it is important to understand the robustness of a method in the hands of different operators. To this end it would be important to at least compare the results of several operators or indeed compare results following technology transfer to an alternative lab. Only one of the records suggest that testing in multiple labs was undertaken whilst none mention multiple operators even within the same laboratory, Supplementary Table 2.

The draft USP guidance suggests that a Validation set be used after model optimisation. This Validation set is entirely different from the Reference set and any samples used in establishing performance criteria. It should be made up equally of Typical and Atypical samples with sensitivity and specificity being determined. Only one publication describes the testing of what could possibly be described as an independent Validation set, Table 2.

The lack of testing on real unknown field samples in many of the publications is a concern, especially in the methods developed using artificially spiked samples, after the method performance has been determined using the Test set, or Validation set. Almost all of the articles reviewed concluded with the description of method performance after testing with Typical and spiked samples. This, in the opinion of the authors does not indicate that the non-targeted method will work in the field. To perform this work may require some form of real sample survey where samples undergo secondary testing to confirm the results from the non-targeted method. Without such work on field samples, the non-targeted method remains a laboratory proof-of-concept.

From the various points outlined, it is clear that a common set of performance characteristics with a common nomenclature and set of definitions needs to be established and adopted in order for non-targeted methods to be developed to the same standard, especially when the point of the research is to do more than show a laboratory based proof-of-concept.

*2.3.3. Databases and sharing of data*

From reviewing the publications no views can be expressed on the collection and storage of metadata related to the samples. As previously mentioned many of the publications use spiked samples. The collection of samples from retail outlets make it extremely difficult to conduct in-depth auditing processes to determine the original source of the samples. Where samples have been collected from producers, more metadata will be available. In the articles, only sample type and location have typically been provided. However authors may have collected more details.

No articles describe the use of databanks developed by other researchers. All describe generating their own databases of spectra and there is no indication that these are being shared. Sharing of course poses its own set of issues. How meaningful will any data generated be without using the same instrumentation and settings unless this was included in the validation exercise? Therefore, a central repository database of metadata and spectra should be considered for non-targeted methods for the detection of food fraud. However, the willingness, by researchers, to not only give up control of all their data but also trust that other researchers have collected data with the same rigor, will need to be addressed. Many researchers in this field choose to generate their own data and gradually update their own databases as all intellectual property rights can be protected. Potential future developments could incorporate the development of Apps that would provide access to databases secured in a cloud based environment. These Apps could facilitate simplified multiuser access and information sharing.

## 3. Hyperspectral imaging

HSI is mostly used in (rapid) detection of defects in and quality of foodstuffs such as meat, fish, fruit, dairy and animal feed (Amigo, Marti, & Gowen, 2013; Baeten, Pierna, & Dardenne, 2007; Dale et al., 2013; Elmasry, Kamruzzaman, Sun, & Allen, 2012; Kamruzzaman, Makino, & Oshita, 2015; Lohumi, Lee, Lee, & Cho, 2015; Qiao, Ngadi, Wang, Gariepy, & Prasher, 2007; Wu & Sun, 2013a, 2013b), or even trace-level detection of peanuts in wheat flour (Mishra et al., 2016). Applications for non-targeted food fraud testing are less numerous, a number of which are listed in Table 3.

Almost all of the hyperspectral methods in Table 3 use a pixel-based approach, which yields a large number of predominantly NIR spectra per sample, but discards the spatial coherence present in the analysis. About half of the studies report calibrations of a specific adulterant rather than broad classification for authentic/adulterated.

Application and certainly validation of this type of analysis is largely uncharted territory. Almost all of the methods in the list are essentially feasibility studies, and not yet meant to be applied in practice. Most use only a limited number of samples, gathered at a single time from a limited number of sources, and thus cannot be expected to cover the natural variation of product and adulterant, which is the main objection to these methods to be used in practice. With additional samples from different sources and time to cover the intended scope, ensuring stable measurements over time, and formulating a scientifically sound mechanism on which hyperspectral imaging might detect the adulteration, a proper validation report could be generated for most of the methods mentioned to be employed in practice.

One exception of the list above is Pierna et al. (Pierna, Dardenne, & Baeten, 2010), which has shown performance equivalent to an existing reference method for a large number of samples, which can be seen as a more direct route to formal validation, and might be considered fit for legal scrutiny in the near future.

**Table 3**
Applications of hyperspectral imaging on food authenticity issues from scientific literature, indicating the method's principle, sample size and validation approach.

| Aim | Method setup | Validation approach | Ref. |
|---|---|---|---|
| *Meat and fish* | | | |
| Freshness, fresh vs thawed pork | NIR HSI with wavelength selection, pixel-based, 24 samples frozen 0–4 times | LOO-CV on 24 samples, 24 independent samples as test set | (Barbin, Sun, & Su, 2013) |
| Speciation between beef, pork, and lamb | NIR HSI, pixel based classification, 225 meat samples (75 for each species) | LOO CV in model phase, 70 additional external validation samples, of which 50% minced | (Kamruzzaman, Barbin, ElMasry, Sun, & Allen, 2012) |
| Detection of horse in minced beef | NIR HSI, pixel based, 38 meat samples (unclear number of sources) | Validation using self-made mixes at different levels, including 13 validation samples | (Kamruzzaman et al., 2015) |
| Detection of beef and pork mixtures | multispectral analysis, pixel based, unknown number of sample sources | "External validation" set is an independent replicate of the mixtures from the training set | (Ropodi, Pavlidis, Mohareb, Panagou, & Nychas, 2015) |
| Detection of gelatine addition to chicken | NIR HSI, pixel-based, 16 chicken carcasses from 2 breeds | Self-prepared additions of hydroxyproline to pieces of each carcass. 46 external validation samples from 4 of the 16 carcasses | (Xiong, Sun, Xie, Han, & Wang, 2015) |
| Detection of gelatine addition to shrimp | NIR HSI, pixel-based, 100 shrimp samples from 5 sources | Self-prepared additions with gelatine, 60 additional (non-spiked) additional control samples | (Wu, Shi, He, Yu, & Bao, 2013) |
| *Fruits and vegetables* | | | |
| Geographical origin of Fuji apples | NIR HSI, based on 207 apple samples | Validation approach unknown | (Guo, Huang, Chen, & Peng, 2013) |
| Geographical origin of rice | NIR HSI, average of rice-pixels. 225 samples from four regions in China. | "Full cross-validation" is reported, but no other validation statements are made | (Kong, Zhang, Liu, Nie, & He, 2013) |
| Detection of *Durum* in *Solstice* Wheat | Feasibility study on 2 samples and their mixtures | No validation statements are made | (Wilkes et al., 2016) |
| Green pea addition to pistachio nuts | Raman HSI, pixel based, 10 pistachio and 10 green pea samples | Validation using self-made mixes at different levels, no further validation | (Eksi-Kocak, Mentes-Yilmaz, & Boyaci, 2016) |
| Coffee variety (Arabica or Robusta) | NIR HSI, pixel based, 18 and 15 green bean samples per variety from a wide variety of sources | Cross validation, PCA + kNN and PLS-DA algorithms both as classical and sparse models, no further validation | (Calvini, Ulrici, & Amigo, 2015) |
| Degree of coffee roasting | NIR HSI, pixel based, 2 batches of 15 brands from light, medium and high degrees of roasting | Cross-validation, including classically derived extractable sugar and protein for classification | (Nansen, Singh, Mian, Allison, & Simmons, 2016) |
| Tea quality estimation | HSI to extract textural features, number of samples unknown | Validation approach not known | (Juanrong & Saritporn, 2008; Zhao, Chen, Cai, & Ouyang, 2009) |
| Tea speciation in tea blends | HSI NIR, pixel based, 3 tea varieties, 3 samples and 18 replicates of each | Cross validation, checking repeatability of replicates, confirmation against UPLC/MS variety-specific compounds | (Djokam, Sandasi, Chen, Viljoen, & Vermaak, 2017) |
| Detection of buckwheat in black pepper | HSI, pixel based after wavelength selection. 4 samples | Additional validation samples, and validation on self-made mixtures | (September 2011) |
| *Others* | | | |
| Melamine in milk powder | NIR HSI, semi-targeted for melamine, pixel-based | In-house validation using self-prepared mixtures from one batch of milk and pure melamine | (Fu et al., 2014) |
| Detection of animal protein in feed | NIR HSI, semi-targeted: specific for animal protein, but different sources | Validation using 85 materials from interlaboratory trials with known reference results. Testing LOD, cross-contamination and (in-house) long term stability using control charts | (Pierna et al., 2010) |
| Melamine and analogues in soybean (feed) | NIR HSI, semi-targeted for melamine and chemical analogues, pixel-based | In-house validation using self-prepared mixtures, using 40 soy bean samples in different forms (meal, hulls, full-fat, dehulled and organic soya) | (Pierna et al., 2014) |

## 4. NMR spectroscopy

NMR spectroscopy is one of the major techniques used for the non-targeted analysis of food for its authentication. Apart from its function as structural elucidator, NMR spectroscopy became and is becoming a more and more interesting tool in metabolomics, nutritional, food science and food authentication also due to its unique quantitative properties. NMR spectroscopy is characterized by an excellent linearity; the generated signals are proportional to the underlying molar concentrations over orders of magnitudes. If quantitative measurements conditions are adjusted each observed atom, independent from its molecular environment, shows the same signal response, allowing the exact cross sample - PULCON method (Wider & Dreier, 2006) or quantitative (qNMR) method to perform substance quantification, even if the respective standard is not available.

The sensitivity depends on the field strength, the type of probe used and the experimental set-up. Sensitivity can be increased by increasing field strength, use of specialist probes (e.g. cryoprobes) or through longer experimental acquisition times. Detection limits are related to absolute molar concentrations of analytes, but for typical 'small molecules' detection limits of 50 $\mu g\,kg^{-1}$ can be achieved with the use of high field, cryoprobe equipped instrumentation coupled with extended acquisition times (Charlton, Donarski, Jones, May, & Thompson, 2006).

Typically, in the case of food and wine analysis, instruments from 400 MHz up to 600 MHz are established. The chemical shift value of an NMR resonance contains information relating to the local chemical environment, therefore the technology is used for structure elucidation of unknowns. Recently - during the last 10 years – quantitative $^1$H NMR spectroscopic methods for fruit juice, and even more recently for wine and honey, have become of interest in research and routine applications.

Instrumental developments enable simultaneous fast and reliable quantification and authentication of food ingredients by the application of multivariate chemometrics to $^1$H NMR data. However, due to its lower spectral resolution, signal overlap occurs in $^1$H NMR spectroscopy and needs to be considered carefully in the spectra evaluation. In addition, it must be noted as general remark that $^1$H NMR is currently not a tool for regular trace analysis. Expected limits of detection for quantification go down to the low mg $l^{-1}$ range for routinely used NMR instruments of the newest generation.

Data acquisition can be completed within a few minutes with a reasonable signal to noise ratio including the detection of minor components. The disadvantages of $^1$H NMR spectroscopy do include the high initial set-up costs, requirement for dedicated housing facilities, supply of cryogens and dedicated expert staff.

The use of $^1$H NMR for quantification has been reported as early as 1963 (Jungnickel & Forbes, 1963), and was recently reviewed as a topic by Bharti and Roy in 2012 (Bharti & Roy, 2012). In relation of food,

**Table 4**
Summary Table collating specific information about manuscripts published between 2011 and 2017 that performed non-targeted analysis of 1H spectroscopic data.

| Matrices analysed | Numbers | Objectives[a] | Relative amounts (%) | Samples used for model creation | Relative amounts (%)[b] | External samples for validation | Relative amounts (%) |
|---|---|---|---|---|---|---|---|
| Alcoholic Beverages | 15.0% | Geographical Origin | 45.0% | < 50 | 45.0% | Not performed | 80.0% |
| Honey | 15.0% | Botanical origin | 37.5% | 50–100 | 25.0% | Yes | 20.0% |
| Vegetable Oil | 25.0% | Production methods | 10.0% | 101–500 | 17.5% | | |
| Spices | 7.5% | Age | 5.0% | > 500 | 10.0% | | |
| Other | 37.5% | Adulteration detection | 25.0% | | | | |

[a] The objectives of several manuscripts had more than one objective.
[b] In one manuscript it was not clear how many samples had been used for model creation.

qNMR methods for specific analytes have been recorded. For example the absolute quantification of methylglyoxal in Manuka was reported by Donarski et al. (Donarski, Roberts, & Charlton, 2010). Furthermore, ring trials for quantitation of multi-component mixtures has been demonstrated using qNMR, demonstrating the phenomenon in complex samples across a range of NMR field strengths (Gallo et al., 2015).

The application of NMR spectroscopy for non-targeted analysis includes several steps. Sample preparation should be as minimal as possible to acquire as many signals as possible. For certain substances e. g. organic acids their chemical shifts show dispersion according to the pH value of the sample (Godelmann et al., 2013), therefore some preparation protocols include the thorough adjustment of the pH in the samples (e.g. for wines). Also algorithms have been developed for the computational corrections of such misalignments (Savorani, Tomasi, & Engelsen, 2010). The acquisition of data has also made tremendous progress during the last decade. Signal suppression of water, and more recently other major solvents has become routine (e. g. ethanol) (Duarte et al., 2002; Monakhova et al., 2011) and the repeatability and reproducibility (Minoja & Napoli, 2014) is improved, setting the prerequisite for databanks of spectral information. After data acquisition a procedure that is called binning or bucketing is often performed, whereby small chemical shift regions (bins or buckets, e.g. 0.1 ppm) are summed together into one new one (Sousa, Magalhaes, & Castro Ferreira, 2013). Thus, the number of variables is reduced and small shifts are equalised. The data matrix then is the input for further multivariate statistical evaluations.

As result of the mentioned advantages and technical progress practical applications using 1H NMR spectroscopy (400 MHz) in the field of food analysis and authentication were developed and are commercially available by the Bruker Corporation. The methodology aims to combine the utility of quantitative NMR with non-targeted analysis using specific experimental acquisition parameters (for qNMR) and reference databases (for non-targeted analysis). Their FoodScreener™ combined with the Profiling™ technique enables the comparison of non-targeted spectral $^{1}$H NMR data with the corresponding group of reference spectra (e.g. database of several thousands of reference fruit juices, wines or honeys, obtained from production sites all over the world) using verification models (Minoja & Napoli, 2014). The aim of the classification analysis in the case of the WineScreener™ for example is the verification of the grape variety, geographical origin and vintage. This is a stepwise process and includes several statistical models which are set-up in a decision tree/cascade. FoodScreener™ concept involves a decentralized sample preparation and subsequent measurement (in the respective laboratory), but data evaluation is performed centralized on one server (Bruker), creating a report, which is send back to the customer. The sample preparation procedure as well as the $^{1}$H NMR measurements are to be performed following a strict protocol. As with all non-targeted analysis issues can arise regarding the nature of the samples stored within the databases, especially when information is held in proprietary databases. A more robust solution is the creation of open access databases.

In addition to the high-field applications ( ≥ 300 MHz) also low field NMR instruments (45–90 MHz) are recently be used for authentication purposes. Only a few applications e. g. on meat or edible oils have been published so far (Jakes et al., 2015; Parker et al., 2014). Processing is different than for high resolution NMR, typically the peak areas of certain signals are considered for the statistical evaluation.

To determine the status of $^{1}$H NMR databases for detection of food fraud in the academic sector, a literature review was conducted using the search terminology of (NMR OR ″Nuclear Magnetic Resonance”) AND (authen* OR adulter*) AND (food OR honey OR oil OR wine OR spice) for peer reviewed articles published between 2011 and 2017. Articles were filtered to those that performed classification of samples using non-targeted analysis and multivariate analysis. In total 40 articles were reviewed to form an overview table that reported the food type analysed, objectives of the manuscript, the number of samples used for model creation and whether an external validation set (defined as samples that were not used in model creation or validation although these could have been collected/prepared at the same time as the samples used in model creation) was used. The results are shown in Table 4.

The availability of external raw data was also reviewed, in all cases raw data was not available for download. Therefore, currently no solutions exists for open access non-targeted analysis databases based on $^{1}$H NMR spectroscopy.

## 5. Conclusions

In order to attempt to keep pace with those who perpetrate food fraud there is clearly a need for robust and reliable non-targeted methods that are available to many stakeholders. Before we can address questions such as their potential use as confirmatory methods, it is important to tackle the challenges the research and routine testing communities faces in terms of having methods which are fit for purpose. Without official guidelines and recognised performance criteria, these non-targeted methods will only ever be considered as screening tools and only those that can prove themselves to function correctly in the field. A number of authors have already demonstrated a 2 tier monitoring system where non-targeted screening tests were developed and ran in conjunction with confirmatory techniques (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016; Wielogorska et al., 2018). This may well prove to be the systems needed to have broad spectrum, cost effective monitoring programmes in place to cover the complex and growing area of food fraud.

### 5.1. Summary of challenges

- A lack of guidelines and legislation governing both the development and validation of non-targeted methodologies
- No common definition of terms leading to difficultly in interpretation of data
- The difficulty in obtaining authentic samples with full traceability for model building
- A lack of guidelines and legislation describing both how spiked samples should be prepared and at what concentrations
- A lack of certified reference materials

- Many users, as well as legislature, view chemometrics as a “Black Box” where there is limited understanding of what is happening “under the lid.”
- Chemometric software is expensive, especially to for the non-academic developer. A more cost effective all-inclusive intuitive package is required.

### 5.2. Summary of recommendations

- Make use of robust and reliable instrumentation
- Develop guidelines and ultimately legislation to standardise language, development and validation procedures
- Adopt a common nomenclature for ease of comparison and interpretation of results
- Develop guidelines and ultimately legislation to outline samples needed to build robust models
- Where possible include certified reference materials in model development and testing/validation sets. (The same sample cannot be use in each).
- Sampling database should be fit for purpose and consist of at least 200 samples for each class
- In order to describe representativeness when talking about total sample numbers, make a distinction between unique samples collected and samples created through spiking
- When preparing spiked samples it is important to replicate the industrial process as closely as possible in order to prepare samples that match those likely to be created at that stage
- The setting of concentrations on preparing spiked samples at various concentrations should be intelligence led. Experimental design should allow the lowest concentration of adulterant to be determined (based on economically motivated fraud i.e. in many instances there is no economic motivation to adulterate at low levels).
- When preparing spiked samples per adulterant, a cross representative selection of authentic samples should be used to spike samples to account for natural variation within the sample.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tifs.2018.04.001.

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